CHAPTER III

MATERIALS AND METHODS

1. Experimental design

In order to study the effect of ERK inhibition on diabetic neuropathy, the animal experiment was designed as in Fig. 2.

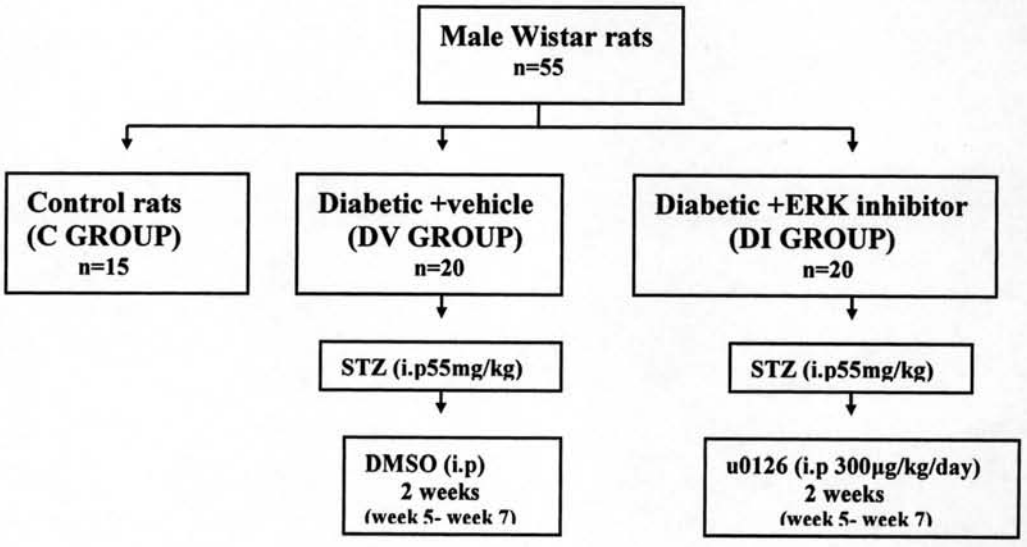

Figure 2: **Experimental design**

In this study, fifty-five male Wistar rats each weighing 250 g were used (from the National Laboratory Animal Center,Mahidol University,Thailand). They were randomly divided into 3 groups:These were control group (C group) (n=15), diabetic-vehicle group (DV group) (n=20) and diabetic-inhibitor group (DI group) (n=20).

The procedures involving animals were approved by the institutional ethical committee and done according to the guideline declared by the National Research Council of Thailand. All efforts were made to minimize animal suffering and to reduce the number of animals used.

1.1 Induction of diabetes

One week was allowed for the animals to settle down. Diabetes was induced by a single intraperitoneal injection of 55 mg/kg body weight of Streptozotocin (STZ) in the DV and DI groups. Both diabetic and non-diabetic rats were group-housed on a 12-h light / 12-h dark cycle and fed standard rat chow and water ad libitum at a room temperature of 25±2°C.

1.2 Confirmation of diabetes

Blood glucose level was measured from tail vein blood using glucose test strips 48 hours after STZ injection. Only animals with blood glucose higher than 250mg/dl were included for the experiment as diabetics (Price SA et al., 2004).

1.3 Drug administration

For drug administration, the C group received no injection. In the DV group, the vehicle, dimethylsulfoxide (DMSO) was injected with the volume calculated according to the body weight equivalent to the DI group. The vehicle was injected intraperitoneally once daily started from week 5 for two consecutive weeks. For the DI group, u0126 (Promega,USA), purchased as dried material, was resuspended with 250 µl of DMSO per one vial (1mg of u0126) to produce a stock solution of 4 µg/µl. The stock solution was kept at -20°c. On the same day prior to injection, further dilution was carried out with DMSO 1:12 (stock:DMSO) to make a final solution for injection 1ml/kg body weight. With this dilution, each rat received 300 µg/kg/day of u0126. U0126 was administered intraperitoneally once daily from week 5 for two consecutive weeks.

The reason of using this dose regimen was that Namura et al., 2001 has found the effective inhibition of ERK using 200 µg/kg/day of u0126 in the animal model of brain ischemia. However, our pilot study did not show the same degree of inhibition in the DRG of diabetic rats, suggesting the higher dose of u0126.

2. Electrophysiological measurement

Introduction

Nerve conduction studies are performed to diagnose disorders of the peripheral nervous system and help delineate the nature and distribution of the neural lesion. With this technique, electrical stimulation of the nerve initiates an impulse, which travels along motor and sensory fibers. The assessment of conduction response depends on analysis of compound muscle action potential (CMAP) recorded from the muscle in the study of motor fibers and nerve action potential in case of sensory fibers. Usually, the nerve conduction velocity (NCV) of the motor and/or sensory fibers was calculated.

Procedure

In this study,the test of motor NCV was performed to assess peripheral nerve function.The two stimulation point method was used (Figure 3). The rats were anesthetized by 4% halothane with 1 l/min oxygen for induction. The concentration of halothane was then decreased to 2 to 2.5% with 500 ml/min oxygen for maintenance of anesthesia. The animal was placed on the hot pack and was covered by a warm blanket. The stimulating needle electrode was placed at left sciatic notch close to the sciatic nerve (Point 1). Then, the active recording and reference recording electrodes were placed at the second and third interosseous spaces of left hind paw (Figure 3).

Finally, the ground electrode was placed between the stimulating and active recording electrodes at lateral side of foot. The sciatic nerve was stimulated at least 4 times with supramaximal stimuli and the electrical activity (M wave) was recorded using the oscilloscope. Then, the stimulating electrode was moved to the Achilles' tendon close to the tibial division of sciatic nerve (Point 2) and the stimulation was repeated. The traces were averaged and used to calculate the NCV. The motor NCV was measured one week before the beginning of the experiment (baseline), and at the end of the experiment at week 7. The stimulation points must be as close as possible to the nerve.

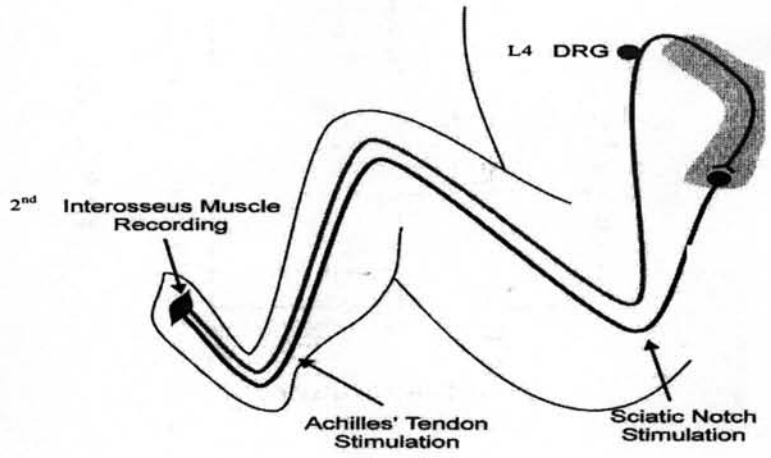

Figure 3 Diagram explaining position of stimulating and recording electrodes in NCV study [Modified from Fig. 1 in Patel and Tomlinson, 1999]

Latency is defined as the time from the stimulation of the nerve to the peak of CMAP. Therefore, in case of stimulation at the two points (point 1 and point 2), L1 and L2 were latencies of the stimulation at point 1 and point 2, respectively (Figure 4).

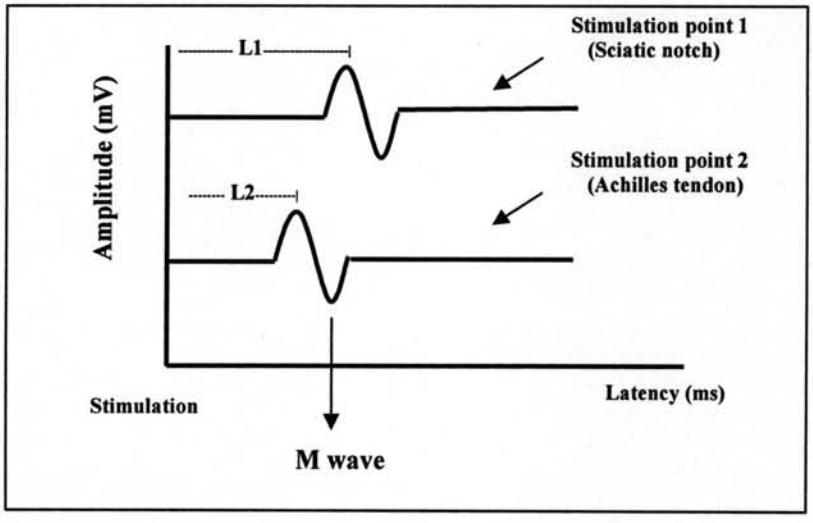

Figure 4 Compound Muscle Action Potential (CMAP) recording after two-point nerve stimulation where point1 is at the sciatic notch and point 2 at Achilles' tendon , L1=latency of the stimulation point1 , L2= latency of the stimulation point 2

The conduction velocity is calculated by dividing the distance between the two stimulation points by the difference between the latencies of the two stimulation points which is the duration the nerve impulse takes to travel between the two stimulation points as shown in the formula below.

$$V\ m/s = \frac{D\ (mm)}{L1\text{-}L2\ (ms)}$$

V = nerve conduction velocity
D = distance between the two stimulation point (point1 and point2)
L1, L2 = the latencies of the stimulation at point 1 and 2 in milliseconds, respectively

3. Tissue collection

3.1 Blood collection for plasma glucose determination

At the end of the experiment, twenty animals(C=8, DV=6, DI=6) were deeply anesthetized by halothane. The chest wall was opened as quickly as possible to expose the heart. Blood was collected by cardiac puncture to the right atrium and stored in the tubes with anticoagulant. The blood tubes were then transferred to the Department of Laboratory Medicine for determination of plasma glucose using the glucose oxidase method.

3.2 Fresh removal for Western blot analysis

After the blood was taken, L4/5 dorsal root ganglia (DRG) were immediately removed and stored in Eppendorf tubes. These tissues were kept at -70°c until use.

3.3 Removal after perfusion for morphological studies

At least 5 animals per group were killed with overdose halothane. After opening the thoracic cavity, the animal was intracardially perfused with 100 ml of normal saline followed by 500 ml of 4% paraformaldehyde (PFA) via a needle in the left ventricle. After perfusion, the L4/5 DRG and sciatic nerve were removed.

4. Western blot analysis

Introduction

Western blot analysis is used to separate the proteins in the samples according to their molecular weights and measure amount of the protein of interest. In this study, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used.

4.1 Sample preparation

L4/5 DRG were homogenized in homogenization buffer [0.1 mmol/l PIPES pH 6.9, 5mmol/l magnesium chloride, 5 mmol/l EGTA, 0.5% Triton X-100, 20% glycerol, 10 mmol/l sodium fluoride plus 1 mmol/l PMSF, 2 mmol/l sodium orthovanadate and protease inhibitor cocktail (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml benzoyl-L-arginine methyl ester, 10 μg/ml p-tosyl-L-arginine methyl ester, 10 μg/ml L-1-tosylamide-2-phenylethylchloromethyl ketone, 10 μg/ml trypsin inhibitor and 10 μg/ml aprotinin); all from Sigma]. Sample buffer (0.25 M Tris pH 6.8, 10% glycerol, 0.01% bromophenol blue, 10 mM dithiothreitol, 2% SDS and 2% β-mercaptoethanol; all from Sigma) was added to the samples before boiling for 5 min. These homogenized samples were stored at -20°C until use.

4.2 Bramhall protein assay

This technique is the Bamberg modification of Bramhall et al., 1969. This method is based on the principle that Coomassie Blue (Coomassie Brilliant Blue G, Sigma, UK) binds to the protein in an acidic medium. The amount of Coomassie dye bound to the sample is relative to the amount of protein in that sample. Thus, the protein concentration in the sample can be determined by comparing with the protein standard.

Procedures

A sheet of Whatman No.1 filter paper was separated into equal squares (1.5 x 1.5 cm). Bovine serum albumin (BSA) protein standard (1 mg/ml, Pierce, UK) was applied on the filter paper from 0.5 to 8 µl in triplicate. Then, 3 µl of homogenization solution (blank) or 3 µl of each sample was applied to the paper in triplicate. The paper was permitted to dry and washed briefly with absolute methanol to remove other substances from the paper which might interfere with the dye-binding reaction. After drying, the paper was stained for at least 30 min in a solution of 0.5 % Coomassie Blue (dilution of 5% stock by 7% acetic acid). Consequently, it was washed with several changes of 7% acetic acid to remove non-specific binding and allowed to dry. The filter paper was then cut into individual square and each square was put in a 2-ml Eppendorf tube. To elute the bound stain, 1.4 ml of elute solution (66% methanol, 33% distilled water and 1% ammonium hydroxide) was added in each tube. The tubes were vortexed, left at room temperature for 15 min and vortexed again to ensure the complete removal of dye stain. Standards, blanks and samples (200 µl) were transferred to a 96-well plate and a plate reader (Multiskan Ex,ThermoLabsystems) was used to read the absorbance at 600 nm.. Values of blanks were subtracted from those of the standards and samples. The values of 9 standards were plotted and a standard curve was obtained. Calculation of protein concentration in the samples was obtained by referring to the standard curve.

4.3 Electrophoresis

Running gel (separating gel, 10% polyacrylamide) and stacking gel were set using Biorad mini-gel apparatus (wide Format Mini COC system). The running gel was prepared first (see the formula in Appendix 1) and N,N,N′,N′-tetramethylethylene diamine (TEMED) was added directly prior to use. TEMED was added to catalyze the release of free radicals from ammonium persulfate (APS) that play a role in accelerating acrylamide polymerization. The running gel was poured into the space between 2 glass plates and allowed to set for at least 15 min. The stacking gel was consequently prepared (see the formula in Appendix 1) and poured on the top of running gel. The comb was directly inserted into the stacking gel and the gel was left to polymerize for 15 min. The comb was removed and the samples were loaded in the volumes equivalent to 10 µg of protein into wells following brief boiling of samples.

Protein markers or standards (Kaleidoscope Prestained Standards, Biorad) were run on one well of the gel. The circuit was completed by adding running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3, Sigma). The voltage was applied at 100 V until the proteins concentrated to form a sharp line and passed into the separating gel. The voltage was then increased to 150 V, and the electrophoresis was run for approximately 60 min.

4.4 Protein transfer to the membrane

Following gel electrophoresis the proteins separated by molecular weights were transferred to nitrocellulose membrane using semi-dry electroblotter (Bio-rad). Four sheets of blotting paper were placed beneath the membrane. Then, the gel was placed on the membrane followed by another 4 sheets of paper. The blotting paper was soaked with the transfer buffer for at least 15 min before use. After assembling the electroblotter, electrical current was set at 0.35 A and run for 10 min (for 2 gels). The membranes (blots) were then removed from the electroblotter and washed briefly with 1% casein solution (Vector Laboratories).

4.5 Immunoperoxidase procedure

Introduction

"ABC Method" is an immunoperoxidase procedure for localizing a variety of antigens. This procedure employs biotinylated antibody and Avidin-Biotinylated enzyme complex and has been termed the "ABC"technique. Because avidin has such an extraordinarily high affinity for biotin, the binding of avidin to biotin is essentially irreversible. In addition, avidin has four binding sites for biotin, and most proteins including enzymes can be conjugated with several molecules of biotin. Therefore, in this study, ABC method was used to detect antigens of interest on the nitrocellulose membrane.

Detection procedure

The ABC detection kit from Vector Laboratories (Vectastain ABC kit) was used. The membrane was transferred to a solution of primary antibody [rabbit antibodies to total or phosphospecific ERK1and 2 (1:500 and 1:5000, respectively, Santa Cruz Biotechnology)] in 1% casein and incubated overnight at 4°C. Different membranes were probed for total ERK (ERK-T) and phosphospecific ERK (ERK-P). Then, the membrane was washed with at least 3 changes of casein 15 minutes with gentle agitation and then incubated in a solution of biotinylated secondary antibody in casein for 30 minutes. After incubation, washing with 3 changes of casein was repeated.

Subsequently, the membrane was transferred to the substrate solution (Appendix 4) [DAB substrate kit for peroxidase (SK-4100), Vector Lab, USA] and incubated at room temperature until suitable staining developed. Development time was generally 1-2 minutes. When staining was satisfactory, the membrane was rinsed in water for 5 minutes and left air dry.

4.6 Densitometric analysis

The membrane was scanned with flat-bed scanner [CANON 9950 F (Canoscan resolution 4800 ×9600 dpi)]. All scanned digital images were imported to microcomputer in tiff format and the densities of specific bands were analyzed with image analysis program (Image ProPlus 4.5). Density of each band was analyzed and the value of the background was subtracted. Ratio of phosphorylated to total ERK was calculated. The data of 2 isoforms of ERK (ERK1 and ERK2 at 44 and 42 kDa, respectively) were combined.

5. Microscopic examination

5.1 Preparation of DRG and sciatic nerve for morphological analysis

After transcardial perfusion, removed sciatic nerve and L4 DRG were further fixed in 3% glutaraldehyde for 24h at 4°C. The samples were rinsed with 0.1 M cacodylate buffer followed by osmification with 1% osmium. Following additional rinse in cacodylate buffer, the nerve samples were dehydrated through ethanol with increasing concentrations followed by propylene oxide: Epon and finally embedded in epoxy resin (Details are shown in Appendix 2). Semithin sections (0.5μm) of sciatic nerve were cut from the epon blocks using glass knives on an ultramicrotome, and then stained with 1% para-phenylenediamine. Thick sections (2μm) of DRG were obtained using an ultramicrotome, and stained with toluidine blue. For preliminary screening of section, the slides were examined under a light microscope at 10x.

5.2 Quantitative evaluation (Morphometry)

5.2.1. Nerve morphometry

In each nerve section, all parameters were counted and calculated using Image-Pro Plus software after the images were imported into a microcomputer (under ×40 objective). Next, the area of nerve fascicle was measured (mm^2) using a magnification of x4. The three windows with an area of 0.012 mm^2 /window (under 40x) were randomly placed on the section. With this strategy, three windows were assumed to represent different areas of fascicle. One window was in the center of the fascicle, while the other two were placed on each side of the central window (Fig.5). The frame of each window must not be in contact with the perineurium or the adjacent window. Axons which were not totally inside the window were discarded. Then, each window was analyzed the number of myelinated axons, myelinated axon diameter, myelinated fiber diameter, *g* ratio, myelin thickness and myelinated fiber density were calculated from the three windows. At the same time, the g-ratio (i.e. the ratio between the axonal diameter and the fiber diameter of the same axon) was calculated (Fig.6). Myelin thickness was calculated as half of the difference between axon diameter and fiber diameter. Axon density was calculated by dividing the total number of myelinated fibers by the fascicle area. Then, the number of axons was extrapolated for the whole nerve by using the ratio of the total window to the total fascicular areas. However, the quantification of total number of fibers, might

calculated back to all fascicle compared with total area of three window and total fascicle area (area/mm^2) divide an area of one three window (0.036 mm^2). The examiner was unaware of the experimental groups during the analysis. Therefore, the results were checked for errors, for example, missed axons or count of small artifacts and appropriate corrections were made by the same examiner.

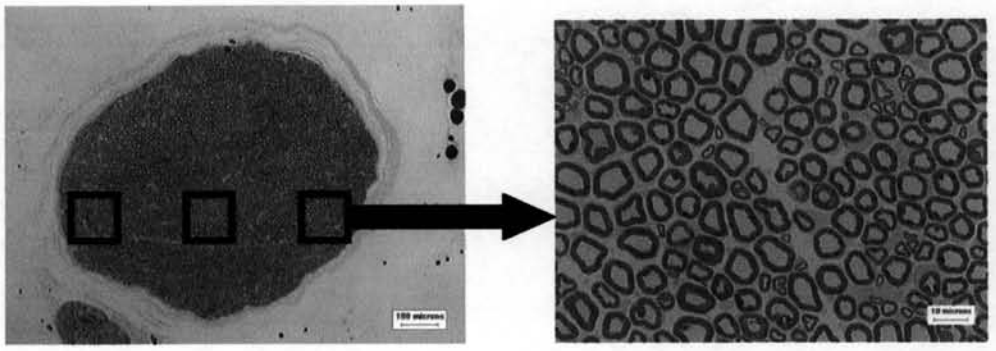

Figure 5 Micrograph showing the placement of three windows within a fascicle. with one window in the central zone. The area of each windowwas 0.012 mm^2. Scale bar represents 10 μm.

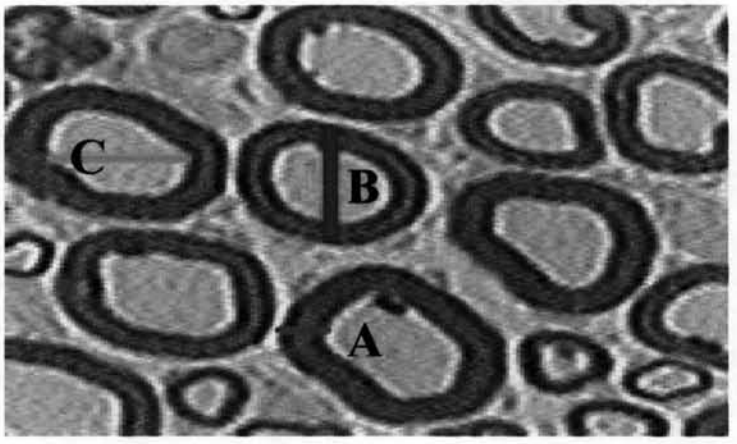

Figure 6 Diagram explaining the myelinated axon ;Myelin thickness (A) , Fiber diameter (B) and Axon diameter (C).

$$\text{g-ratio} = \frac{\text{Axonal diameter (C)}}{\text{Fiber diameter (B)}}$$

$$\text{Myelin thickness (μm)} = \frac{\text{Fiber diameter (B) - Axon diameter (C)}}{2}$$

$$\text{Fiber density (myelinated fibers/mm}^2\text{)} = \frac{\text{Total myelinated fibers}}{\text{Fascicle area}}$$

5.2.2 DRG Morphometry

Glutaraldehye-fixed, resin-embedded L4 DRG were serially cut into 2μm thick sections and stained with toluidine blue. The first section to be used for neuron counting was randomly chosen from 1-9. Then, every neuron with visible nucleus and nucleolus in every 20 th section (40μm apart) (Fig. 7) (for example 5th, 25th, 45th, 65th...etc) was counted using photoshop program. The total number of neuron/DRG was then calculated.

$$\textbf{Total no.of DRG neuron} = \frac{\textbf{No. of neurons in the sample sections X Total no. of section}}{\textbf{No. of sample sections}}$$

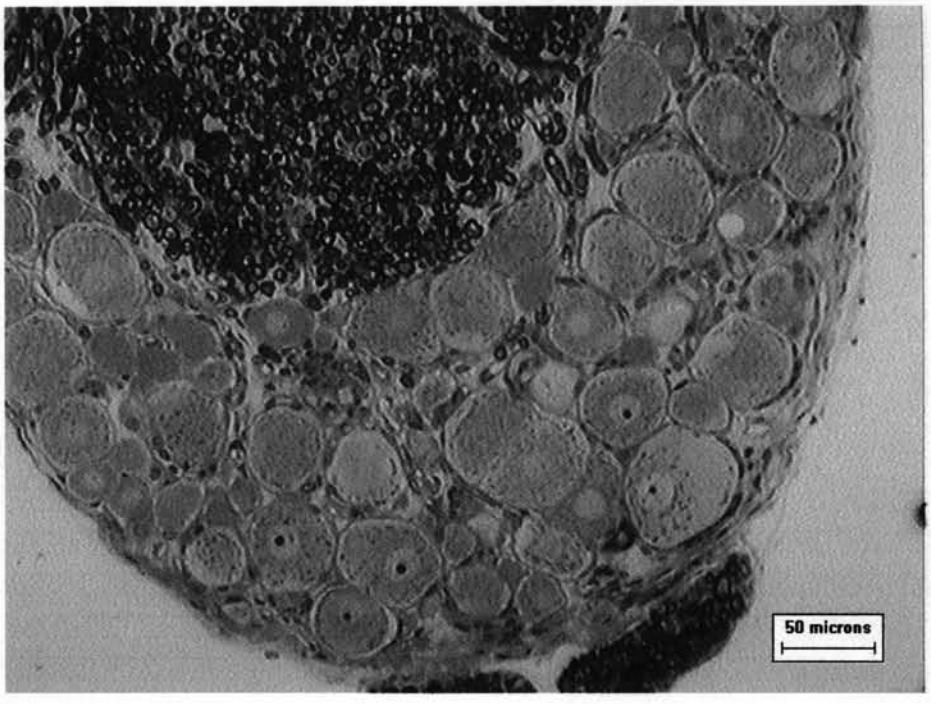

Figure 7 Micrograph showing L4 DRG neurons stained with toluidine blue (10x).

Furthermore, areas of the nucleus and nucleolus were measured in 300 randomly selected neurons using Image Pro Plus Software.

6. Statistical analysis

Comparison between 3 independent groups

All data were analyzed using SPSS for Windows version 11 for statistical analysis. The data were checked for normal distribution and homogeneity of variances. Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. However, if the measurements were extremely skewed from normal distribution and/or had a markedly significant difference in variances, a Kruskal-Wallis test (non-parametric test for ANOVA) was used. Statistically significant differences were shown when p values were less than 0.05, unless otherwise stated.